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ANTIBACTERIAL ACTIVITY (ANTI-ADHERENCE AND ANTI-BIOFILM) OF PLANTS EXTRACTS AGAINST BLACK-PIGMENTED BACTERIA (*PREVOTELLA INTERMEDIA*) : AN *IN VITRO* STUDY IN HILLA CITY, IRAQ

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ABSTRACT : To evaluate the antimicrobial and antibiofilm activity of plant extract (*Origanum majorana*, *Salvia rosmarinus*, *Ceratonia siliqua*, *Plantago Ovata* and *Senna acutifolia*) against oral Pathogens *Prevotella intermedia* by an *in vitro* method. All tested isolate were susceptible to plant extract (*Origanum majorana, Salvia rosmarinus*, *Ceratonia siliqua*, *Plantago Ovata* and *Senna acutifolia*) with variable degrees of antimicrobial inhibition. The extract was effective against *P.intermedia* with inhibition zone ranging from 32 – 24mm. The extract represented as a potent anti-biofilm agent with dual actions, preventing biofilm formation and also eradicating the existing biofilm.

Key words : Antibacterial activity, anti-adherence and anti-biofilm, plants extracts, black pigmented bacteria (*Prevotella intermedia*).

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INTRODUCTION

Dental plaque is a biofilm that evolves normally on teeth. It involves of clusters of 500 to 600 various bacterial taxa established in a matrix of polymers of bacterial and salivary source (Socransky and Haffajee, 2002). Dental plaque in good health persons, stays constant for long times in order to a dynamic equilibrium among the static members of its microbial community (Marsh, 1989). The disease appears when the microbial homeostasis during the plaque breaks down to disarrangement of the natural element's ecology (Marsh and Bradshaw, 1997). In periodontal disease, there is a shift in the composition of subgingival plaque's microflora that establishes a colony in tooth exterior and epithelial cells in the periodontal pocket to an additional proteolytic gram-negative anaerobic groups involving the pigmented rods in the genera *Porphyromonas* and *Prevotella* (Moore and Moore,1994; Socransky *et al*, 1998).

Black-pigmented anaerobes such as *Prevotella intermedia* have been engaged as pathogens related with the beginning and fabrication of periodontitis (Dahlén, 1993; Haffajee and Socransky, 1994; Haffajee *et al*, 1998; Ximenez-Fyvie *et al*, 2000; Ximenez-Fyvie *et al*, 2000). These species be based largely on superficial heme as an iron source for their growth (Okamoto *et al*, 1998) and accumulate a cell surface black pigment that mainly consists of μ-oxobisheme of iron protoporphyrin IX (PpIX) and monomeric iron PpIX (hematin) in *P. intermedia* (Smalley *et al*, 2003). *P. intermedia* is a black-pigmented anaerobic Gram-negative bacterium, it is related with oral diseases, such as chronic periodontitis (Ashimoto *et al*, 1996; Petit *et al*, 1994; Polson *et al*, 1997). Attacker periodontitis (Albandar *et al*, 1997; Kuru *et al*, 1999; Kamma *et al*, 2004), adulthood-related gingivitis, sever necrotizing ulcerative gingivitis (Rowland *et al*, 1993; Novak, 1999), periapical periodontitis(Gomes *et al*, 1994; Jacinto *et al*, 2003) and sever gangrenous disease (Bolivar, 2012; Falkler *et al*, 1999).

In addition to being implicated in oral diseases, *P. intermedia* has also been notified to be related with

various systemic diseases, such as cystic fibrosis, chronic bronchitis (Shinzato and Saito, 1994; Ulrich *et al*, 2010; Brook and Frazier, 2003) and atherosclerosis (Fiehn *et al*, 2005). Hardness in preventing the bacterium has been referred to impedance of *P. intermedia* to many antibiotics, including penicillins, cephalosporins, and tetracyclines (Andrés *et al*, 1998; Fosse *et al*, 2002). Furthermore, *P. intermedia* cells form a biofilm in which the bacterial cells become more resistant to antibiotics, biofilm can work for a container of antibiotic resistance (Takahashi *et al*, 2006). So, it is of clinical importance to evolve another antimicrobial oncoming for prevailing antibiotic-resistant *P. intermedia*.

The seeds/shell of *Plantago ovata* include hemicelluloses, carbohydrates, protein, tannin, glycosides, fixed oil, linoleic acid, palmetic acids etc. Medical utilizes for treatment constipation, dysentery, intestinal ulcers, peptic ulcers, diabetes mellitus, hyperlipidemia (Abdul Nasir *et al*, 2004). *Senna acutifolia* generally utilized in Mexico for treatment patients with diabetes millets type 2 (Raffoul-Orozco *et al*, 2017). The carob (*Ceratonia siliqua* L.) is a leguminous plant that retains green leaves throughout the year, shrub or tree. Carob powder is a natural sweetener, with a flavor and appearance similar to chocolate; therefore, it is often used as a cocoa substitute, free of caffeine and theobromine. Furthermore, the carob has vitamins A and B and some substantial metals, such as K, P, Ca and Mg, as main metals and Fe, Mn, Zn and Cu as trace metals (Soliman and Jacob, 2010).

Salvia rosmarinus (rosemary) is an odorous evergreen sapling, have several of bioactive components mostly inclusive polyphenols, phenol diterpenes and triterpenes. Rosmarinic acid, carnosic acid, carnosol, caffeic acid, betulinic acid and ursolic acid are the predominant constituents. *S. rosmarinus* is also a rich origin of fundamental oil (Loizzo *et al*, 2013). The fundamental oil of *S. rosmarinus* was demonstrated to have antibacterial, antioxidant, antifungal and anti-flaming features (Ka¡cániová *et al*, 2017). *Origanum majorana* have two various chemo types are known. The oil of the primary chemotype is rich in monoterpene alcohols - terpinen-4-ol, cis-sabinene hydrate, trans-sabinene hydrate and α-terpineol. The oil of the other chemotype has the phenolics thymol and carvacrol as main components ((a) Lawrence, 1979; (b) Lawrence, 1991; (b) Lawrence, 2000; (c) Lawrence, 2004; Baratta *et al*, 1998). Fundamental oils from both chemotypes are applied in perfumery, cosmetics and food industry. The oil from the latter chemotype distinguishes itself by a profound antiseptic action, due to the high concentration of thymol and carvacrol (Hiroe and Nobuji, 1993).

In the present study, we have evaluated the antimicrobial and anti-biofilm activity of plant extract (*Origanum majorana*, *Salvia rosmarinus*, *Ceratonia siliqua*, *Plantago ovata* and *Senna acutifolia*) against oral pathogens *P. intermedia* by an *in vitro* method.

MATERIALS AND METHODS

For preparation of Aquatic Extract from *Origanum majorana*, *Salvia rosmarinus*, *Ceratonia siliqua*, *Plantago ovata* and *Senna acutifolia*. Related Aquatic Extracts Table 1 according to Hindi (2013).

Table 1 : Herbal extracts used in the study.

Part used	Common name	Scientific name
Seed	Psyllium	*Plantago ovata*
Leaf	Senna	*Senna acutifolia*
Seed	Khurnub	*Ceratonia silique*
Leaf	Rosemary	*Salvia rosmarinus*
Leaf	Marjoram	*Origanum majorana*

Bacterial and isolates

A 50 samples were taken from pockets of patients with periodontic; 30 male and 20 female, the people from twenty to sixty six years recent (periodontic Department, Teaching Clinics of Oral and Dental Surgery). Thereafter, cultivated on blood agar plates now incubated aerobically and anaerobically (within the anaerobic jar) at 37°C for twenty four to seventy two hours and 10% CO_2. Then subjected to identification per the cultural properties such as black pigmented colonies, microscopic examination such as capsule and organic chemistry tests enzyme, biochemical tests catalase, Indole, antibiotic (vancomycin) sensitivity (30µg) and vatic test (Forbes *et al*, 2007).

Antimicrobial activity test by Agar-well diffusion assay (*In vitro*) : According to Hindi *et al* (2013, 2014).

Antibacterial activity assay : According to Forbes *et al* (2007) the antimicrobial activity was detected by agar-disc diffusion (the test were performed in triplicates).

Biofilm formation assay : Semi quantitative microtiter plate test or Tissue culture plate method assay (TCP) designated by Hindi *et al* (2016) was assumed as the gold standard method for detection of biofilm formation (Table 2).

Table 2 : Bacterial adherence and biofilm formation by method of TCP.

Mean of OD value at 630 nm	Adherence	Biofilm formation
0.120<	Non	Non
0.240- 0.120	Moderately	Moderate
>0.240	Strong	High

Adherence test

Bacterial adherence to epithelial cell of the mouth is one of the main and important virulence properties of these bacteria and can be identified using method designated by Mateveki *et al* (2004) and Avila-Campos *et al* (2000).

Statistical analysis

Bonferroni test was used to analyses data; as (P 0.05) to show significant differences between the types of extracts (Danial, 1988).

RESULTS AND DISCUSSION

In this study, 50 Samples were collected from pockets of patients with periodontic; 30 male and 20 female, the people from twenty to sixty six years recent, only 8 sample of *P. intermedia*, while other sample distribution between *Strep. mutanus*, *Staph. aureus*, *Bacillus* spp. and *Porphyromons gingivalis*.

The important biochemical test were used to detection the *P. intermedia* are tabled in the Table 3 also detected by the vitek 20 assay.the important characteristic of the *P. intermedia* as a black pigmented colonies, convex, 1-2 µm in diameter, after 72hr. The microscopic morphology are Gram negative coccobacilli surrounded with a capsule or hallow. Positive for Indole test and Lipase, while negative for Urease and Catalase test.Vancomycin sensitivity are resist.

Porphyromonas–Prevotella group includes non-motile, non-sporing, black pigmented Gram-negative coccobacilli. Even though they are commensals of oral cavity, many species are associated with a variety of orodental as well as invasive human infections (Beena *et al*, 2017).

Antimicrobial activity test of plants extracts

Plant extracts are one of the most important alternatives to antibiotics. It has been used for a long time, primarily because of its ease of use and cheapness, in addition to its availability and high effectiveness in eliminating microorganisms. Through this research, results revealed the efficiency and effectiveness of the extracts in eliminating some types of bacteria that cause Orodental infections as well as invasive human infections. As these isolates are exhibiting resistance to many antibiotics, an attempt is done in this study to evaluate antimicrobial activity of various herbal extracts from natural sources. All tested of *P.intermedia* was susceptible to all plant extract with variable degrees of inhibition zones.

Table 3 : The biochemical test, appearance and microscopic morphology of *P. intermedia.*

Biochemical tests	Result
1-vancomycin sensitivity	Resistance
2- Urease test	-
3- Lipase	+
4-Catalase test	-
5-Indole	+
Appearance on blood agar	Characteristic after 72hr black pigmented colonies, convex, 1-2 µm in diameter
Microscopic morphology	Gram negative coccobacilli surrounded with a capsule or hallow

The extract was found high effective figure No.1.the highest inhibition zone of *Ceratonia silique* (32mm), *Salvia rosmarinus* and *Senna acutifolia* (30mm), *Origanum majorana* (28mm) then *Plantago ovate* (24mm).

The current results of the plant extracts (*Origanum majorana*, *Salvia rosmarinus*, *Ceratonia siliqua*, *Plantago Ovata* and *Senna acutifolia*) are showed a high efficiency in inhibiting *Prevotella intermedia* bacteria compared to the traditional antibiotics (Figs. 1 and 2). In contrast to traditional antibiotics, Impenium is appeared higher inhibition zone about (22mm), fallowing erythromycin with inhibition zone (18mm) and finally amoxicillin and cefotaxin with inhibition zone (12mm).

On the other hand, the extract represented as a potent anti-biofilm agent with dual actions, preventing biofilm formation and also eradicating the existing biofilm, range from high to moderate action. We noted in Table 4, the *Salvia rosmarinus*, *Senna acutifolia* and *Origanum majorana* had high activity against *P. intermedia*, while *Ceratonia siliqua* and *Plantago ovate* had moderate action against *P. intermedia*.

The action of plant extract led to the secondary metabolites. After the development of every organisms, plants have these days progressed their molecular antimicrobial strategies to remain alive, by making secondary metabolites with synergistic action like small antimicrobial peptides, alkaloids, coumarins, flavonoids, phenols, phenolic acids, quinones, saponins, tannins and terpenoids (Breen *et al*, 2015; Gyawali and Ibrahim, 2014). For instance, the two major compounds of *Origanum*, namely thymol and carvacrol, display synergistic action against *P. intermedia* (Didry *et al*, 1994; Hernández-Hernández *et al,* 2014).

The main phytochemicals discovered in *C. siliqua* L. are polyphenols containing concentrated and hydrolysable tannins, phenolic acids, flavonoids and flavonoidal glycosids indicating a potency antibacterial

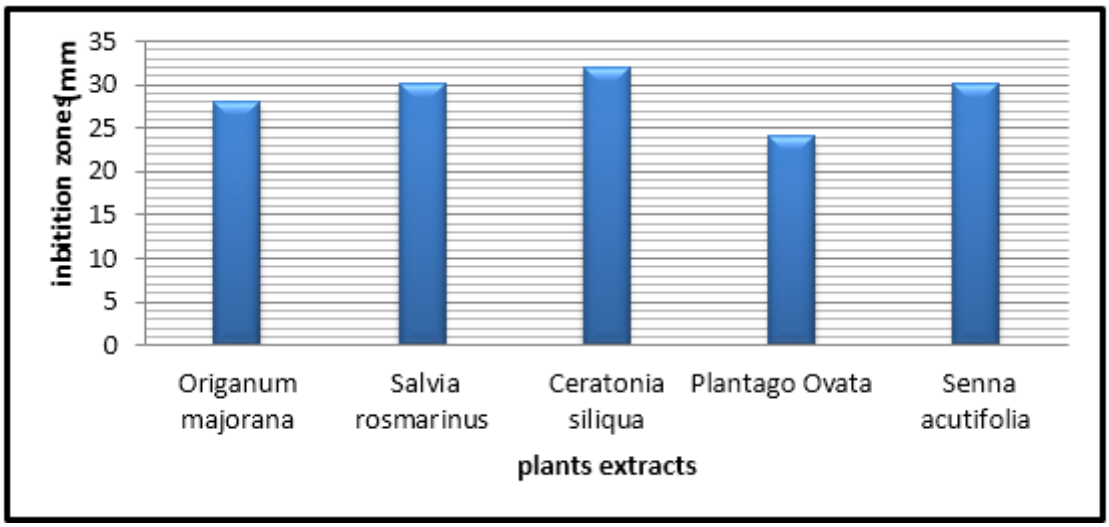

Fig. 1 : Graph showing zone of inhibition of *Origanum majorana*, *Salvia rosmarinus*, *Ceratonia siliqua*, *Plantago Ovata* and *Senna acutifolia* against *Prevotella intermedia* bacteria by agar well method.

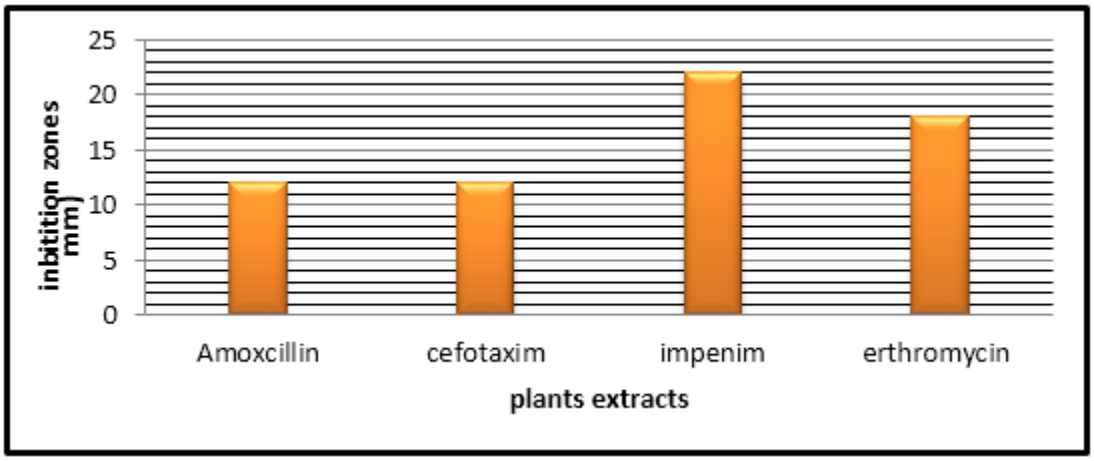

Fig. 2 : Graph showing zone of inhibition of Antibiotics against *P. intermedia* bacteria by disc well method.

Table 4 : Anti-adherence activity & antibiofilm activity of aquatic extracts of *Origanum majorana*, *Salvia rosmarinus*, *Ceratonia siliqua*, *Plantago ovata* and *Senna acutifolia* against *P. intermedia* bacteria.

Plant extracts	Adherence	Biofilm formation
Salvia rosmarinus	High	High
Ceratonia silique	Moderate	Moderate
Plantago ovata	Moderate	Moderate
Senna acutifolia	High	High
Origanum majorana	High	High

and cytotoxic actions (Owen *et al*, 2003; Avallone *et al*, 1997; Nachtomi and Alumot, 1963; Henis *et al*, 1964; Kivçak *et al*, 2002; Tassou *et al*, 1997; Custódio *et al*, 2011; Ben Hsouna *et al*, 2011). Separated secondary metabolites are predestined to be <10% of the overall number obtainable in plants. These metabolites are mostly utilized as protection mechanisms against insects, herbivores, and microorganisms. The broad diversity comes from the plants' capacity to synthesize a large arsenal of aromatic compounds and their oxygen-substituted derivatives (Cowan, 1999). For example, *S. rosmarinus* includes a wide spectrum of plant secondary metabolites. Aqueous extracts include large condensations of flavonoids and phenolic acids. *S. rosmarinus* extracts demonstrate bactericidal and bacteriostatic actions against both Gram-positive, Gram-negative species, antifungal and antiviral effectiveness have also been notified (Al-Juraifani, 2009; Ghorbani and Esmaeilizadeh, 2017). And for *S. acutifolia*, the medicinal properties are expected to their contents of hydroxyanthraquinone derivatives and contain of numbers powerful purgatives activities (Shyamala and Thangaraju, 2012). The antibiotic influence of *P. ovata* extract may be connected to their secondary metabolites (Windisch *et al,* 2014). Therefore, these extracts are active against *P. intermedia* bacteria which cause dental carries, and more efficient in discouraging it than traditional antibiotics. However, further studies are needed for the definitive administration of these extracts as the source of antibiotic plants.

CONCLUSION

A plant extract (*Origanum majorana*, *Salvia rosmarinus*, *Ceratonia siliqua, Plantago ovata* and *Senna acutifolia*) possesses compounds with good antimicrobial properties that may be used for oral infectious diseases caused by certain oral pathogens associated with dental caries and/or periodontal diseases. For the application, a plant extract may be incorporated in mouthwash or toothpaste.

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